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# Biodistribution Analysis of Oncolytic Adenoviruses in Patient Autopsy Samples Reveals Vascular Transduction of Noninjected Tumors and Tissues

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In clinical trials with oncolytic adenoviruses, there has been no mortality associated with treatment vectors. Likewise, in the Advanced Therapy Access Program (ATAP), where 290 patients were treated with 10 different viruses, no vector-related mortality was observed. However, as the patient population who received adenovirus treatments in ATAP represented heavily pre-treated patients, often with very advanced disease, some patients died relatively soon after receiving their virus treatment mandating autopsy to investigate cause of death. Eleven such autopsies were performed and confirmed disease progression as the cause of death in each case. The regulatory requirement for investigating the safety of advanced therapy medical products presented a unique opportunity to study tissue samples collected as a routine part of the autopsies. Oncolytic adenoviral DNA was recovered in a wide range of tissues, including injected and noninjected tumors and various normal tissues, demonstrating the ability of the vector to disseminate through the vascular route. Furthermore, we recovered and cultured viable virus from samples of noninjected brain metastases of an intravenously treated patient, confirming that oncolytic adenovirus can reach tumors through the intravascular route. Data presented here give mechanistic insight into mode of action and biodistribution of oncolytic adenoviruses in cancer patients.

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## INTRODUCTION

Oncolytic viruses are emerging as a treatment option for cancer with two positive phase 3 trials now completed, one with an adenovirus and another with a herpes virus.<sup>1,2</sup> Overall, the safety of many types of oncolytic viruses including adenoviruses has been demonstrated in a range of trials and evidence of efficacy

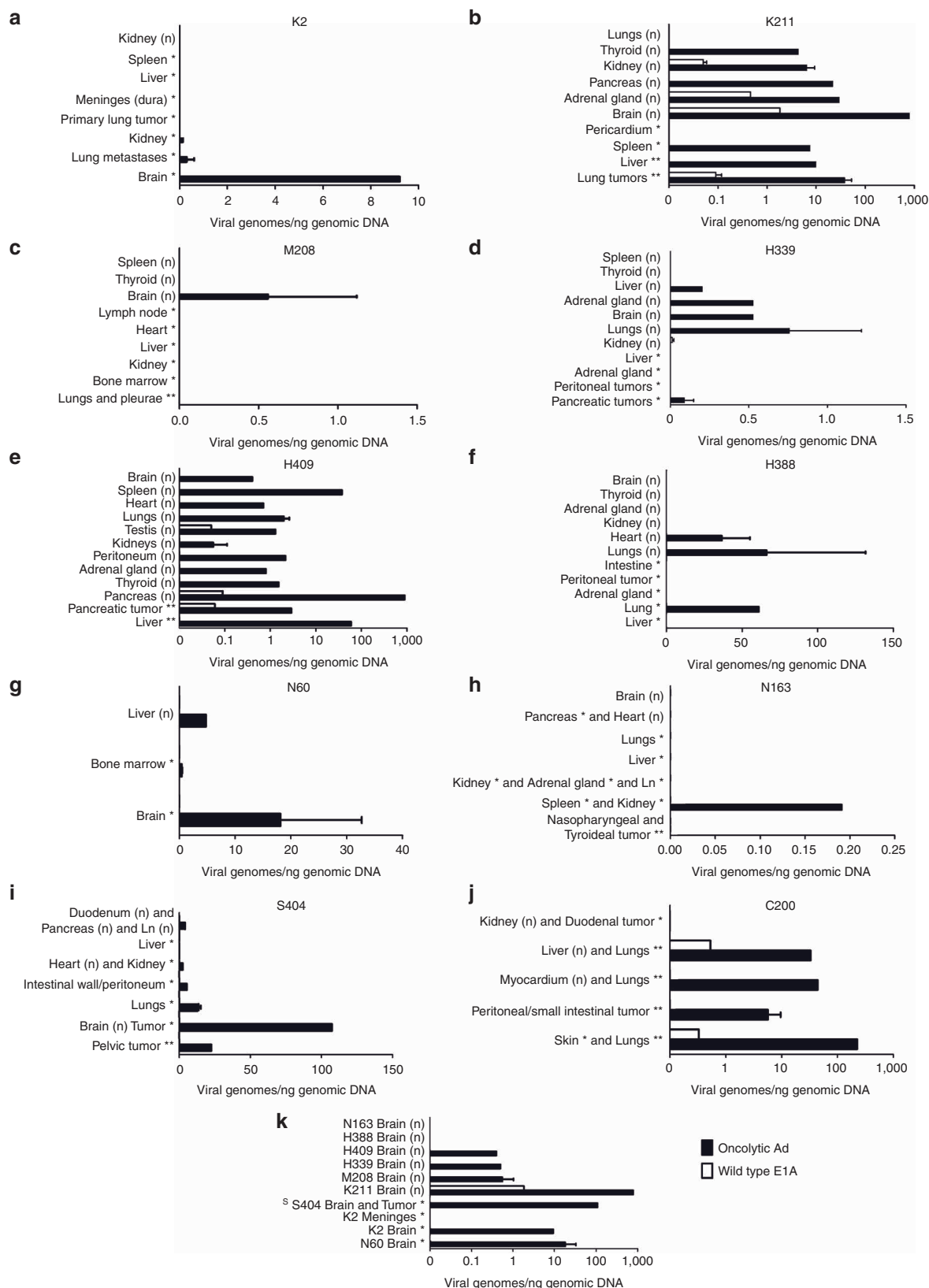
is mounting.<sup>3–9</sup> In particular, virus vectors armed with immunostimulatory molecules are showing great promise in the field.<sup>5,10–13</sup> However, knowledge on the spread and action of the viruses after administration relies mainly on studies in rodents. And while adenovirus biodistribution and kinetics in rodents are thoroughly characterized, these are unlikely to represent the situation in human patients very well, as rodents are not natural hosts of human adenoviruses. Thus far, the available data of oncolytic adenovirus spread and functionality in human subjects consists of detecting virus in body fluids such as blood, ascites, urine, and saliva<sup>6,9,14</sup> and a few individual reports on tumor biopsies taken from patients after treatments.<sup>15–17</sup>

In this patient series, we describe unique human data gathered from autopsies of cancer patients who died as a result of disease progression after they had received oncolytic adenovirus treatments in the Advanced Therapy Access Program (ATAP). According to pathologists' reports, no mortality was attributable to virus treatment, which is in accord with published oncolytic adenovirus trials. Out of 290 treated patients, 11 autopsies (3.8%) were performed to study the cause of death, which was cancer progression in all cases. We felt it was of importance to document these cases in intricate clinical and biological detail. Moreover, tissue samples routinely collected in autopsies and stored in pathology archives would allow us to increase our understanding on biodistribution and mechanism of action of oncolytic adenoviruses. Biodistribution patterns in normal tissues were studied as well as the ability of the virus to transduce distant tumor sites. We also gathered evidence demonstrating the functionality of the virus in noninjected tumor tissues.

## RESULTS

### Oncolytic adenoviral DNA is present in a wide range of normal and neoplastic tissues following virus injections into tumors, body cavities, and vasculature

All day 0 serum samples were negative for oncolytic adenovirus DNA. On day 1 or during the first week after treatment, a burst of viral copies was frequently observed in the serum with eventual



**Figure 1** Oncolytic adenovirus DNA is recovered from a wide range of injected and noninjected neoplastic and normal tissues. **(a-j)** DNA was extracted from paraffin embedded tissue samples collected in autopsies and qPCR was performed with primers and probes detecting genetic modifications of the used oncolytic viruses (black bars) and wild-type E1A region (open bars) and normalized with the  $\beta$ -actin housekeeping gene. **(k)** All samples containing normal or metastatic brain tissue. (n), histologically normal tissue; Ln, lymph nodes. \*Tissue with tumor/metastasis, not injected directly; \*\*injected tumor/metastasis tissue.  $n = 1-15$  samples per tissue, expressed as mean + standard error of mean for tissues with multiple samples.

disappearance within a variable time (Table 2). For most patients, a clear increase was seen between day 1 and a subsequent sampling time, suggesting viral replication.<sup>18–20</sup> In patients who received multiple treatment rounds, nonfirst treatments were generally also accompanied by bursts of viral copies in serum. All serum samples were negative for wild-type adenovirus E1A.

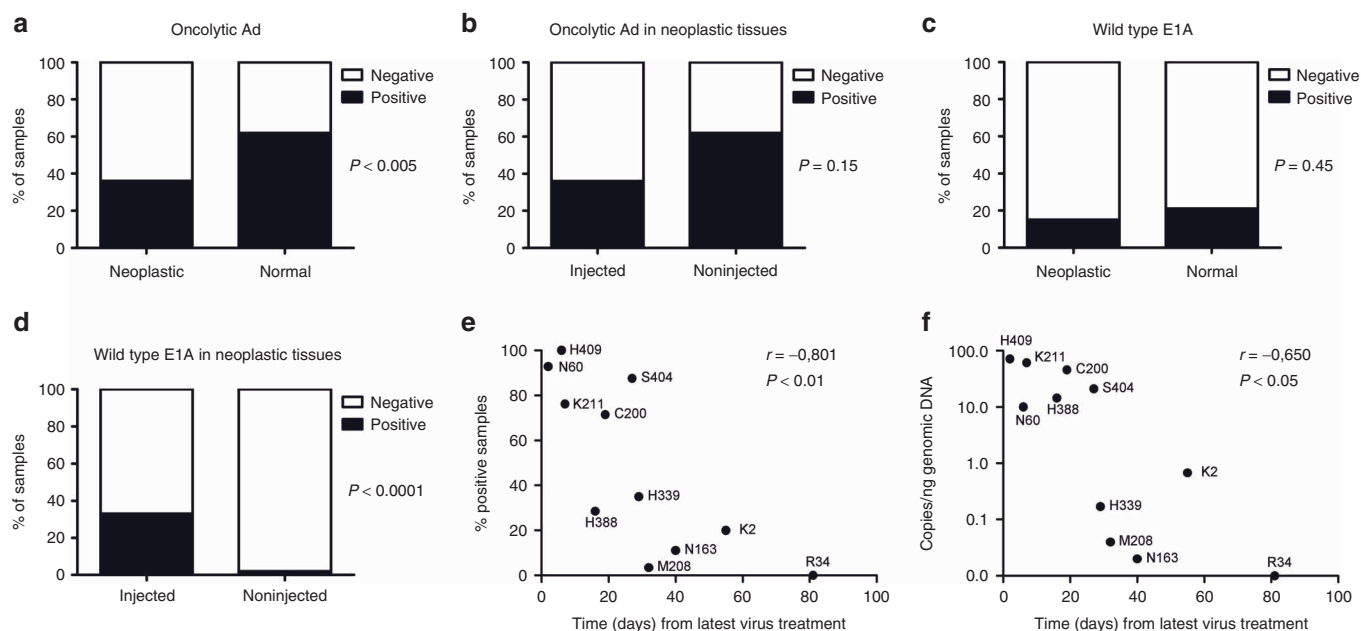
In a pulmonary adenocarcinoma patient (K2) treated with an intravenous bolus and direct virus injections into cutaneous metastases on the scalp, oncolytic adenovirus DNA was detected in the brain parenchyma and also in noninjected lung and kidney metastases (Figure 1a). In another patient with a widely metastatic lung adenocarcinoma (K211), oncolytic adenovirus DNA was detected in all autopsy tissue specimens except the tumor-free lung tissue and metastasis-bearing myocardium (Figure 1b). Highest virus copy numbers were recovered from a sample of the tumor-free brain (frontal cortex). In addition, large amounts of virus genomes were found in lung tumor samples. Wild-type adenoviral DNA was recovered from lung tumor tissue, histologically normal adrenal gland, brain, and kidney. In a mesothelioma patient (M208) treated twice with Ad5/3-D24-GM-CSF, only one sample of brain (cerebral cortex) without metastasis had measurable, low quantities of oncolytic adenoviral DNA (Figure 1c).

In a pancreatic adenocarcinoma patient (H339), treated with intraperitoneal and intravascular injections, oncolytic adenovirus DNA was detected in both the pancreatic tumor specimens and several normal tissues (lung, liver, brain, and adrenal gland) (Figure 1d). Copy numbers recovered from the pancreatic tumor samples were generally low, and five out of the eight examined tumor specimens were negative for adenoviral DNA. Wild-type adenovirus DNA was recovered in minimal quantities

in one histologically normal kidney sample. Another pancreatic adenocarcinoma patient (H409) had experienced worsening of his general condition already prior to the second virus treatment and died two days after the second treatment, due to cancer progression as confirmed by the autopsy. Serum copy numbers had remained quite low during the entire treatment period (Table 2). Nevertheless, oncolytic adenoviral DNA was recovered from all examined tissues (Figure 1e), with moderate copy numbers in the injected pancreatic tumor tissue and liver metastases, and highest copy numbers in tumor-free pancreatic tissue. Wild-type adenovirus DNA was recovered from neoplastic and tumor-free pancreatic tissue and the testes. A third pancreatic adenocarcinoma patient (H388) had twice received treatment with Ad5/3-hTERT-CD40L, by intratumoral injections to liver metastases and also an intravenous bolus. Oncolytic adenovirus DNA was detected in tumor-free samples of myocardium and lung as well as a sample from a noninjected lung metastasis (Figure 1f).

A lobular breast carcinoma patient who died 81 days after her last virus treatment (R34), had shown a notable increase in circulating virus amounts during the first week post-treatment, followed by a decrease and eventual disappearance of virus in the serum (Table 2). Tissue samples from lungs, kidneys, heart, and liver were negative for presence of adenoviral DNA (data not shown).

A young patient with a metastatic neuroblastoma (N60), who had been treated with Ad5/3-Cox2L-D24 intravenously due to the lack of injectable tumors, showed a remarkably high oncolytic virus titer in the serum on day 1 post-treatment (Table 2). Oncolytic adenovirus DNA was detected in all tissue specimens obtained from the autopsy, *i.e.*, a metastasis-free liver specimen as



**Figure 2** Frequency of positive findings and amount of viral DNA decrease with time from treatment to autopsy. (a,b) Percentage of analyzed samples that were positive/negative for oncolytic adenovirus DNA. (a) All samples ( $n = 153$ ) and (b) samples with tumor or metastasis ( $n = 106$ ). (c,d) Percentage of analyzed samples that were positive/negative for wild-type adenovirus E1A DNA. (c) All samples ( $n = 132$ ) and (d) samples with tumor or metastasis ( $n = 94$ ). (e) Time from latest viral treatment and percentage of samples collected in autopsy, which were positive for oncolytic adenovirus DNA ( $n = 3$ –29 sample blocks analyzed per patient). (f) Time from latest viral treatment and average oncolytic adenovirus DNA copy number normalized for  $\beta$ -actin housekeeping gene in all analyzed samples.  $r$ : Pearson coefficient for correlation with time;  $P$ : significance level of correlation.

Table 1 Characteristics of patients and virus treatments

Patient ID	Sex	Age	WHO <sup>a</sup>	Tumor type	Spread of metastases (as determined in autopsy)	Previous treatments	Virus treatments <sup>b</sup>	Sensitizers <sup>b</sup>	Dose <sup>b</sup>	Route <sup>b</sup>	Target of intra-tumoral injection	Signs of treatment effects	Survival (days) after first virus treatment	Survival (days) after latest virus treatment	Cause of death
K2	M	48	2	Lung adenocarcinoma	Lungs, pleurae, spine, scalp/skin, liver, spleen, kidney, brain parenchyma and meninges.	Radiotherapy, cisplatin+docetaxel, penmetrexed, erlotinib	Ad5/3-Cox2L-D24		2 × 10 <sup>9</sup>	50% i.t. 50% i.v.	Cutaneous scalp metastases	Reduction in size of scalp metastases	55	55	Cancer
K211	F	63	2	Lung adenocarcinoma	Lungs, pleurae, mediastinal lymph nodes, esophagus, spleen, mesenterium, liver, spine, pericardium.	Penmetrexed+cisplatin, erlotinib, radiotherapy	Ad5/3-D24-GMCSF	Cyclophosphamide p.o.	3 × 10 <sup>11</sup>	33% i.p., 20% i.p., 27% i.t., 20% i.v.	Tumors in lung, liver and skin		7	7	Cancer
M208	M	62	2	Mesothelioma	Lungs, pleurae and thoracic wall, liver, kidneys, diaphragm, peritoneal lymph nodes, spine, heart.	Penmetrexed+cisplatin, penmetrexed, penmetrexed+carboplatin	Ad5/3-D24-GMCSF	Cyclophosphamide i.v.	3 × 10 <sup>11</sup>	80% i.t., 20% i.v.	Pleural tumors		53	32	Cancer
H339	M	65	1	Pancreatic adenocarcinoma	Liver, adrenal gland, peritoneum, peritoneal lymph nodes.	Gemcitabine+cisplatin, gemcitabine chemoradiation, erlotinib+capecitabine	Ad5/3-E2F-D24-GMCSF	Cyclophosphamide p.o. and temozolomide	8 × 10 <sup>11</sup>	80% i.p., 20% i.v.			29	29	Cancer and acute myocardial infarction
H388	M	66	1	Pancreatic adenocarcinoma	Liver, lungs, adrenal gland, peritoneum, peritoneal lymph nodes.	Surgery, gemcitabine, gemcitabine+cisplatin, capecitabine+oxaliplatin	Ad5/3-hTERT-CD40L		1.5 × 10 <sup>12</sup>	80% i.t., 20% i.v.	Liver metastasis	Progression in CT 7 days from latest treatment	37	16	Cancer
H409	M	57	2	Pancreatic cancer	Liver	Gemcitabine+cisplatin, gemcitabine, oxaliplatin+capecitabine	Ad5-D24-GMCSF	Cyclophosphamide p.o.	3 × 10 <sup>11</sup>	80% i.t., 20% i.v.	Pancreatic tumors and liver metastases		51	2	Cancer
R34	F	59	2	Lobular breast carcinoma	Peritoneum, liver, lungs, kidneys.	Surgery, radiotherapy, mitoxantrone+vinorelbine +carmofur+calcium folinate, letrozole, docetaxel +gemcitabine, faslodex, novantrone+interferon, trofosamide +etoposide, bevacizumab+docetaxel +faslodex+letrozole	Ad5-D24-RGD	Cyclophosphamide p.o.	2 × 10 <sup>11</sup>	65% i.p., 2% i.t., 33% i.v.	Liver metastasis		130	81	Cancer
							Ad5/3-Cox2L-D24	Cyclophosphamide p.o.	2 × 10 <sup>11</sup>	100% i.p.					

Table 1 Continued on next page

Table 1 Continued

Patient ID	Sex	Age	WHO <sup>a</sup> type	Spread of metastases (as determined in autopsy)	Previous treatments	Virus treatments <sup>b</sup>	Sensitizers <sup>b</sup>	Dose <sup>b</sup>	Route <sup>b</sup>	Target of intra-tumoral injection	Signs of treatment effects	Survival (days) after first virus treatment	Survival (days) after latest virus treatment	Cause of death
N60	M	6	2	Neuroblastoma (stage IV)	Brain and spinal cord, bone marrow (spine, femur), peritoneal lymph node.	Surgery, radiotherapy, novantrone+navelbine+mirafur+antrex, letrozole, docetaxel+gemcitabine, faslodex, novantrone+interferon, trofosamide+etoposide	Ad5/3-Cox2L-D24	Cyclophosphamide p.o.	1.5 × 10 <sup>11</sup>	100% i.v.	Radiological PD in brain metastases 5 days post-tr.	6	6	Cancer
N163	M	58	1	Nasopharyngeal carcinoma	Bone marrow, kidneys, spleen, pancreas, adrenal gland, liver, lungs, pleurae, thyroid gland, multiple lymph node sites.	Chemo-radiotherapy with cisplatin, docetaxel+ cisplatin+fluorouracil, boron neutron capture therapy	Ad5/3-D24-GMCSF Ad5-D24-RGD-GMCSF Ad5-D24-GMCSF	Cyclophosphamide p.o. Cyclophosphamide p.o. Cyclophosphamide p.o.	2 × 10 <sup>11</sup> 9 × 10 <sup>11</sup> 2 × 10 <sup>11</sup>	83% i.t., 17% i.v. 92% i.t., 8% i.v. 92% i.t., 8% i.v.	Enlargement of nasopharyngeal tumor and metastatic lymph nodes	102	40	Cancer
S404	F	5	2	Rhabdomyosarcoma	Kidney, pleurae, lungs, mediastinum, peritoneum.	Ifosfamide, vincristine, dactinomycin, doxorubicin, two surgeries, carboplatin, etoposide, thiotepa, intensive treatment with autologous stem cell transplantation, radiotherapy, thalidomide, etoposide, celecoxib	Ad5/3-E2F-D24-GMCSF Ad5/3-E2F-D24-GMCSF Ad5/3-E2F-D24-GMCSF	Cyclophosphamide p.o. Cyclophosphamide p.o. Cyclophosphamide p.o.	7 × 10 <sup>11</sup> 7 × 10 <sup>11</sup> 7 × 10 <sup>11</sup>	80% i.t., 20% i.v. 80% i.t., 20% i.v. 100% i.t.	PMD in PET-CT 15 days after last virus treatment	83	27	Cancer
C200	M	61	3	Small intestine carcinoma (jejunum)	Peritoneum, peritoneal and horacal lymph nodes, lungs abdominal skin,	Surgery, palliative radiotherapy, irinotecan+capecitabine, oxaliplatin	ICOVIR-7		1 × 10 <sup>12</sup>	100% i.t.	Lungs, mesentery, inguinal lymph nodes and iliac bone	19	19	Cancer

F, female; GM-CSF, granulocyte macrophage-colony stimulating factor; i.p., intraperitoneally; i.pl., intrapleurally; i.t., intratumorally; i.v., intravenously; M, male; PD, progressive disease; PET-CT, positron emission tomography-computed tomography; PMD, progressive metabolic disease; p.o., per orally.

<sup>a</sup>Baseline status according to WHO performance criteria. <sup>b</sup>For patients with more than one virus treatment, each treatment cycle is displayed on separate rows.



**Table 2** Viral copy numbers and GM-CSF concentration in patient serum samples during treatment periods

Patient	GM-CSF armed virus(es)	Virus titer in serum (VP/ml) (days from the first treatment)										GM-CSF concentration in serum (pg/ml) (days from the first treatment)							
		0	1	2-7	8-14	21	22	28	29	32-49	≥50	0	1	2-7	14-21	22-32	49	50	
K2	No	0	0	123	0							0.4	7.4	15.3	18.1				
M208 <sup>a</sup>	Yes		125		162		115	0											
H339	Yes	0	136	580								0	0	4.2					
H388 <sup>a</sup>	No	0	0			313	0												
H409 <sup>b</sup>	Yes	0	62			0	0			0	72	0.5	4.5		0.3	0.4	0.3	0.5	
R34 <sup>a</sup>	No	0	130	4820	65					30	0	8.6	1.2	8.3	2.9		7.5	10.0	
N60	No	0	1.27×10 <sup>7</sup>									4.1	9.7						
N163 <sup>b</sup>	Yes		149									5.8	5.0	8.2					
S404 <sup>b</sup>	Yes	0	305	3377				0	40	0	0								

GM-CSF, granulocyte macrophage-colony stimulating factor.

Patients treated with GM-CSF armed virus(es) are indicated. No data from serum samples available for patients C200 and K211.

<sup>a</sup>Patients M208 and H388 had received a second virus treatment 21 days and patient R34 49 days after the first treatment. <sup>b</sup>Patients H409, N163, and S404 received a second treatment 21, 27, and 28 days after the first treatment and a third treatment 49, 62, and 56 days after the first treatment, respectively.

well as tumor metastases in the bone marrow and brain metastases, with highest copy numbers present in the latter (**Figure 1g**). Wild-type adenovirus DNA was not recovered from the tissue specimens.

A patient with a widely metastatic small intestine carcinoma (C200) was treated with intratumoral injections into multiple tumor sites. Oncolytic adenovirus DNA was detected in all investigated tissues except one sample containing tissue from the metastasis-free kidney and a duodenal metastasis (**Figure 1h**). The highest copy numbers were recovered from a paraffin block containing samples of lung and skin metastases. In addition, wild-type adenoviral E1A was detected in two paraffin blocks containing lung and skin metastases and lung metastasis and metastasis-free liver specimens, respectively.

Nasopharyngeal carcinoma patient N163 was treated with virus injections into the primary tumor, cervical lymph node metastases and intravenously. Oncolytic adenovirus DNA was detected in low copy numbers in metastatic tissue samples from the spleen and kidney (**Figure 1i**).

A young patient with a pelvic rhabdomyosarcoma (S404), treated with intratumoral injections to the pelvic tumor and intravenously, exhibited viral DNA in the serum, with a notable increase in viral copy numbers during the first week after treatment (**Table 2**). Oncolytic adenoviral DNA was recovered in all examined tissue sample blocks, except one sample of liver with metastasis (**Figure 1j**). Highest copy numbers were recovered in a sample block that contained tissue from normal brain parenchyma and—confusingly—tumor tissue from an unknown location.

### Transduction of brain tissue was observed frequently

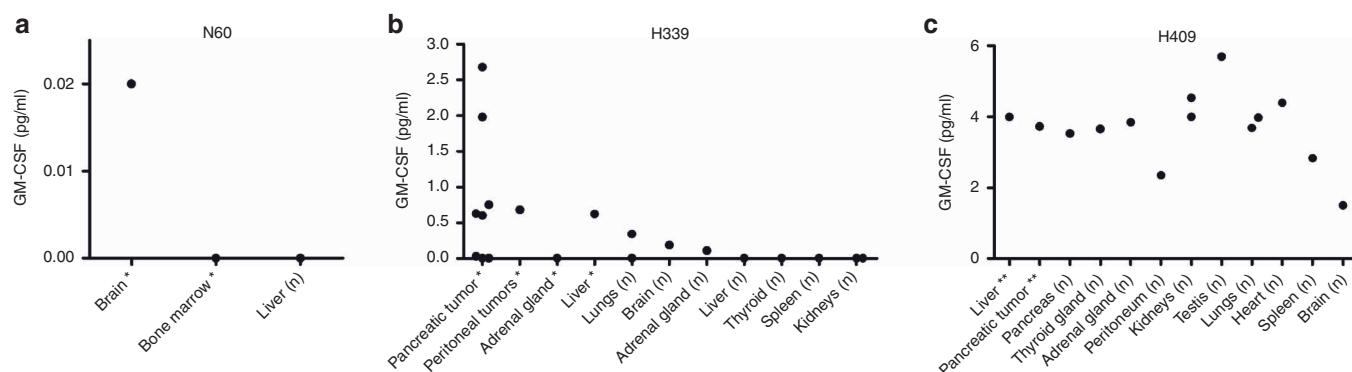
Transduction of brain tissues with oncolytic adenoviruses injected elsewhere was of special interest as it is a safety concern. Oncolytic adenovirus activity in a tissue with cancer may cause inflammatory swelling and edema.<sup>21–24</sup> In the brain, this could be harmful as the brain is confined inside the inflexible skull. Two patients in this series had tumor metastases in the brain (patients K2 and N60). In

both cases, the patients were treated with Ad5/3-Cox2L-D24 and oncolytic adenoviral DNA was recovered from brain parenchyma containing cancer cells (**Figure 1k**). In the case of patient N60, we were also able to recover viable virus from two cryo-preserved tissue samples through standard commercial virus culture methods. Importantly, even though patient N60 died of progression of the brain metastases, there were no unusual signs of inflammation in the tissue as determined by a neuropathological examination performed at a different hospital and independently from personnel responsible for cancer treatment.

Brain tissue samples from seven other patients with no evidence of brain metastases were also examined. For one patient (S404), the brain sample had been embedded together with a sample from a separate tumor from an unknown site, and this paraffin block had moderately high copy numbers of oncolytic adenovirus. For two patients (H388 and N163), the brain sample was negative for adenoviral DNA. The other four patients all had detectable numbers of oncolytic adenovirus copies in the normal brain tissue (**Figure 1k**), and in one out of these patients (K211) we also detected wild-type Ad E1A in the brain sample.

### Oncolytic adenoviral DNA can be recovered from injected and noninjected tumors

In summary, 67 (44%) out of the 153 tissue samples collected from autopsies tested positive for oncolytic adenoviral DNA. A comparison of normal ( $n = 47$ ) and neoplastic tissues ( $n = 106$ ) showed a significant difference: 36% of samples that contained neoplastic tissue were positive for oncolytic Ad DNA, whereas 62% of samples from normal tissues were positive for oncolytic Ad DNA ( $P < 0.005$ ) (**Figure 2a**). Out of the 106 neoplastic tissue samples, 42 samples were from tumor tissues that had been injected (it should be noted that exact correlation of the injection site and autopsy sampling site was not always possible) and 64 from noninjected tumor sites. Interestingly, of these, 45% of injected and 30% of noninjected tumor samples were positive for oncolytic Ad DNA ( $P = 0.15$ , nonsignificant) (**Figure 2b**).



**Figure 3** Human granulocyte macrophage-colony stimulating factor (GM-CSF) levels in cryo-preserved autopsy tissue samples. Human GM-CSF levels were measured from available cryo-preserved tissue samples collected at autopsies of patients A) N60, B) H339 and C) H409.

Twenty-two (17%) of tissue samples, but none of the serum samples, were positive for wild-type E1A. Wild-type adenoviral E1A was detected in 15% of samples with neoplastic tissue and 21% of normal tissue samples ( $P = 0.45$ , nonsignificant) (Figure 2c). Out of the neoplastic tissues, 13 (33%) injected samples and 1 (2%) noninjected sample were positive for wild-type Ad E1A ( $P < 0.001$ ) (Figure 2d).

### Adenoviral copy numbers in tissues decrease with time

As the post-treatment survival time of the patients was variable, the autopsy tissue specimens reflect a variable time span after treatment. Also, types of metastatic tumor and treatment routes were variable, which renders the direct comparison of the cases difficult. Nevertheless, we observed inverse correlations between the time from latest virus treatment and both the percentage of tissue samples positive for oncolytic adenovirus DNA (Pearson coefficient  $-0.801$ ,  $P < 0.01$ ) (Figure 2e) and the mean virus copy numbers detected in the tissues (Pearson coefficient  $-0.650$ ,  $P < 0.05$ ) (Figure 2f). In particular, after 1 month post-treatment, there is a notable decrease both in the frequency of virus positive tissues and the mean virus copy numbers in tissues.

### Human tissues positive for viral DNA do not express detectable amounts of viral hexon

We attempted to do immunohistochemical staining for adenoviral E1A with commercially available antibodies, but were unable to establish a protocol which would work on paraffin-embedded tissues (data not shown). Therefore, we established an immunohistochemical staining for adenoviral hexon, the major capsid protein of the virus (Supplementary Figure S1). In none of the investigated tissues (from patients K2, N60, N163, K211, H339, H388, S404, H409) was there evidence of adenoviral hexon antigen expression (data not shown). In addition, the lung sample from patient S404 had been routinely analyzed by the respective pathology department at the time of autopsy for the presence of adenoviral antigen, also with negative results. These data are compatible with low sensitivity of the assay in the context of human cancer patients.

### GM-CSF

Six patients had received treatment with viruses armed with human granulocyte macrophage-colony stimulating factor (GM-CSF) (Table 1). Serum samples were available for the determination

of GM-CSF levels for three of these patients (N163, H339, H409, Table 2). For comparison, GM-CSF levels were analyzed in serum samples of three patients (K2, R34, N60) that had not been treated with GM-CSF encoding viruses (Table 2). Interestingly, in patients treated with unarmed virus, the mean pretreatment GM-CSF concentration was 4.36 pg/ml and post-treatment 8.92 pg/ml ( $P = 0.21$ ), while it was 2.09 and 2.61 pg/ml respectively ( $P = 0.80$ ) in patients treated with GM-CSF armed viruses. As there were no significant differences in serum GM-CSF concentrations, there was no conclusive evidence of GM-CSF expression. However, if there was expression, it appears to have been restricted to tumor site, which is in line with previous preclinical observations and human data.<sup>1,17,18,20,25</sup>

For patients N60, H339 and H409 cryo-preserved tissues samples from the autopsies were available. Patient N60 was treated with Ad5-Cox2L-D24, which has no transgene. The patient had 4.13 pg/ml GM-CSF in the serum prior to treatment, and 9.65 pg/ml at day 1 post-treatment (Table 2). The tissue sample from the tumor-free liver had no measurable GM-CSF content, and the one from the brain metastasis contained only trace amounts (0.02 pg/ml) (Figure 3a).

For patient H339, treated with Ad5/3-E2F-D24-GMCSF, serum samples from day 0 and 1 had no detectable GM-CSF, but on day 6 post-treatment, 4.23 pg/ml of GM-CSF was detected in the serum (Table 2). In most normal tissue samples (6 out of 8), GM-CSF was not detected, whereas small amounts were found in most (8 out of 11) examined primary tumor or metastases samples (0.02–1.8 pg/ml) (Figure 3b).

Patient H409 received three cycles of treatment with Ad5-D24-GMCSF. On day 1 post-treatment, the GM-CSF level was 4.55 pg/ml and on other measurement days (0, 20, and 21), the level was approximately 0.5 pg/ml (Table 2). GM-CSF was detected also in all tissue samples, at levels between 1.5 and 6 pg/ml (Figure 3c). This patient had received her latest treatment only 2 days prior to her death and virus DNA had also been detected in all organs. Since autopsy tissues will have contain some blood it cannot be excluded that virus and/or GMCSF detected in autopsy were actually present in the blood and not in the tissues.

### Viable virus can be recovered from non-injected metastases

For patient H388, virus culture had been performed by an independent pathology department as part of the autopsy work-up,



from samples taken from the liver and the colon, both with metastatic lesions. These cultures were negative.

Virus cultures were performed by a commercial laboratory from all available cryo-preserved tissue samples (patients N60, H339 and H409). Cultures from patients H339 and H409 were negative, both from tumor-free and neoplastic tissues. For patient N60, virus cultures from the histologically tumor-free liver sample and a sample of the bone marrow with neoplastic cells were negative. Interestingly, viable adenovirus was recovered from two of the three brain metastasis samples. The cultures were then subjected to quantitative polymerase chain reaction (qPCR) analysis and they were found to contain both the treatment virus and wild-type adenovirus E1A DNA. Both samples were also strongly positive with both Cox2L and fiber knob 3 primers, which confirmed that the intravenously administered Ad5/3-Cox2L-D24 virus was present in the tissue.

## DISCUSSION

Predicting when the life of advanced cancer patients ends is clinically demanding because many patients are in relatively good health even a few days before and thus the date of death can only be known afterwards.<sup>26,27</sup> Heavy treatment of patients who are about to die of disease progression should be avoided, but because of the often unpredictable course of the disease it is not rare for cancer patients to receive anti-cancer agents within their last weeks of life.<sup>28,29</sup> Similarly in ATAP, a small proportion of patients died within days to weeks of their latest virus treatments. Overall 3.8% of ATAP patients were autopsied. In less than half of these the death had occurred within 4 weeks of latest virus treatment. We felt that it was important to gather and publish the unique data that was obtained from the autopsies of these patients in order to broaden our understanding of these novel therapeutics. In all cases, the cause of death was determined as disease progression by the independent pathologist performing the autopsy.

Oncolytic adenoviral DNA was discovered in a wide variety of tissues including injected and non-injected sites. At this point, it is unclear if virus DNA present in non-injected organs originates from the initial virus dosing or from progeny virions escaping from injected tumors postreplication. It is also possible that the virus detected in tissue samples was in fact trapped in blood vessels rather than in tissue parenchyma. However, with the possible exception of H409, this seems unlikely as with most patients it was observed that viral DNA had already disappeared from the serum, while we recovered it at high titers from tissue samples. Alternatively the virus might have bound to red blood cells in blood. With our detection method, discordance between serum samples and blood clots, which include the red blood cells, has been shown to occur only in 11% of cases.<sup>19</sup> Therefore, while this might occasionally occur, perhaps in tissues that had very low viral titers and where the time from disappearance of virus from serum to time of autopsy was not very long, it is unlikely to account for majority of the data. Unfortunately, we were not able to determine which cell types the viral DNA was residing: tumor cells, parenchymal tissue or for example tissue macrophages. This aspect would be most interesting to study further, if suitable tissue samples could be obtained. For example immunohistochemistry for adenoviral proteins could be performed or

electron microscopy. These aspects could be incorporated in trial protocols including an autopsy plan.

In addition to metastases in internal organs, oncolytic virus DNA was recovered also from brain metastases of an intravenously treated patient. Thus, this data set provides evidence that oncolytic adenovirus can indeed spread to noninjected neoplastic tissues through the intravascular route. Moreover, as fully replication competent, infectious and viable virus was cultured from the brain metastases of N60, this is evidence of virus replication in a human tumor treated through the intravenous route. One could speculate if replication competent virus could remain from the initial injection, without actual *de novo* replication, but this seems unlikely since virus would have had to remain dormant but infectious for 6 days while it is known that adenovirus loses its infectiveness by shedding its capsid upon entering cells.<sup>30</sup> Thus, it is more likely that the recovered functional virus had indeed replicated in tumor cells.

In addition to brain metastases, oncolytic adenoviral DNA was recovered from four of the six examined tumor-free brains. All four patients had been treated with 5/3 chimeric viruses. The two patients, where we did not detect virus in the brain, had received treatment with Ad5/3-HTERT-CD40L (patient H388) and a serial treatment of (i) Ad5/3-D24-GMCSF, (ii) Ad5-D24-RGD-GMCSF, and (iii) Ad5-D24-GMCSF (patient N163). Therefore, while they had also received a 5/3 chimeric viruses, in the first case, the virus construct otherwise was quite different and in the second the chimeric virus had been administered more than 100 days prior to autopsy. These data are compatible with a hypothesis that 24 bp deleted 5/3 chimeric adenoviruses might be more neurotropic than Ad5 backbone viruses but since we did not have brain samples from patients that had not been treated with Ad5/3 viruses, this remains speculation. Importantly, there were only few neurological adverse reactions in treated patients in this series (**Supplementary Table S1**), and the same is true for previously reported patients,<sup>17,18,20,25,31–34</sup> indicating that brain transduction with oncolytic adenovirus seems safe.

Quantitative PCR does not indicate viable viruses, or virus replication, it merely detects genomes. It is critical to keep in mind that the selectivity of the oncolytic adenoviruses used here occurs postentry and prereplication, meaning that it is expected to recover virus DNA in normal cells. With this in mind, it becomes logical that virus genomes were often found in normal tissues at higher copy numbers than in tumors. In the latter, oncolytic viruses are able to replicate, lysing the cell, releasing the virus from the tissue. If the cells allowing replication die, releasing the virus progeny, they cannot harbor virus and thus will not result in qPCR signals. In contrast, transduced normal tissues will have virus DNA, before being disposed of by DNA damage repair mechanisms and the innate immunity.<sup>35,36</sup> As qPCR analyses were performed on multiple different tissue types, it is possible that the intrinsic properties of the tissues can affect the qPCR reaction and its specificity and sensitivity. Therefore direct quantitative comparisons of viral titers in different tissues may not be reliable. Instead, the qPCR data should be considered qualitative.

We analyzed samples also from one patient whose autopsy was conducted at a notably later time point after the virus treatment, over 80 days later. In this case, no adenoviral DNA was recovered

in any of the samples, suggesting eventual clearance of the virus. Indeed, there was a trend of inverse correlation with time from latest virus treatment to death and mean or median viral copy numbers or the percentage of positive findings in the analyzed samples.

Surprisingly, wild-type adenoviral DNA (wild-type E1A) was recovered in some of the tissue samples, but none of the serum samples. In accord with the serum findings here, also in our previous data, wild-type adenoviral DNA has not been recovered in any serum samples.<sup>17,18,20,25,31–34,37</sup> qPCR for wild-type E1A was positive in a total of 22 analyzed sample blocks (16%). For these tissues, all except one had also been positive for the modified E1A gene, with at least 25-fold higher copy numbers, indicating the presence of higher amounts of the oncolytic virus. Interestingly, wild-type E1A DNA (and oncolytic virus DNA) was encountered also in typing of the viable replication competent virus cultured from the noninjected brain metastases.

It is not clear what these results mean. One, although unlikely, alternative is mutation of the modified E1A region back to the wild type. Adenovirus is a stable double-stranded DNA virus and to our knowledge mutation of a “delta 24” type E1A back to wild-type E1A has not been reported, nor is it easy to come up with a mechanism for it unless wild-type virus would be present in the same cell. Even in this scenario, the presence of GM-CSF would select against this mutation since the transgene renders the virus more immunogenic. Thus, we believe that the finding of wild-type E1A in tumors may represent activation of latent earlier natural adenovirus infection. Adenoviruses have been proposed to exhibit considerable persistence and latency following an acute natural infection.<sup>38–41</sup> We have seen previously that treatment with an Ad3 vector can boost serum levels of Ad5 DNA, indicating reactivation of oncolytic virus from previous treatments.<sup>37</sup> Therefore we believe that the observations of wild-type adenovirus DNA may represent a similar phenomenon, resulting of reactivation of wild-type Ad replication following treatment with an oncolytic virus.

Alternatively, the detection of wild-type E1A could indicate concurrent wild-type adenovirus infection. Since wild-type E1A was not seen in blood, tumors may be preferred locations for wild-type adenovirus DNA because of their immune privileged nature. Yet another explanation could relate to adenovirus sequences reported present in human tumors and also normal cells and tissues, with unclear implications.<sup>39–41</sup> Human adenovirus can transform rodent cells at least *in vitro*, because E1A is expressed in the absence of productive replication, but this seems unlikely to occur in human tissues where the virus can replicate and where antiadenoviral immunity is generated.<sup>42</sup>

Keeping in mind that qPCR does not differentiate between adenoviral sequence integrated into human genomes and episomal virus genomes, and that there is always the possibility of some technical issue, the data should be interpreted cautiously. However, we feel our finding is potentially important and warrants further investigation, and should certainly be kept in mind when interpreting qPCR data in adenoviral gene therapy trials.

In our virus constructs, GM-CSF transgene expression is linked to activation of the viral E3 promoter and thus mostly associated with virus replication.<sup>17,18,20,34,43</sup> We have shown previously

in animal models that virally produced human GM-CSF, or other transgenes under the same promoter, do not leak significantly from the tumor site to the vasculature<sup>18,44</sup> In previously published patient data from ATAP, there have not been significant elevations in serum GM-CSF levels of patients treated with GM-CSF encoding adenoviruses.<sup>17,18,20,25</sup> Likewise, in this series, serum GM-CSF levels did not exhibit major peaks, although in a few patients there was a slight elevation during the week after treatment, compatible with the maximum of virus replication. Overall, GM-CSF viruses did not result in significantly higher GM-CSF concentrations than seen in patients treated with viruses lacking GM-CSF arming. Therefore, there was no conclusive evidence of GM-CSF expression. However, if there was expression, it appears to have been restricted to the tumor site, as reported previously.<sup>17,18,20,25</sup> This is an important safety aspect as the useful effects of transgenic GM-CSF would be predicted to occur in the tumor microenvironment, while adverse effects could result from systemic levels capable of recruitment of myeloid suppressor cells.<sup>13</sup>

GM-CSF measurements from cryo-preserved samples can be considered indicative of functionality of the virus in tissues, although admittedly it is not possible to assess if the detected GM-CSF is endogenous or virally produced. Unlike for serum samples, GM-CSF concentrations in tissues of patients H339 and H409, treated with GM-CSF encoding viruses, were higher than of patient N60, treated with Ad5/3-Cox2L-D24, lacking any transgene. Also, for patients H339 and H409, there was a trend of higher GM-CSF concentrations and more frequently positive samples in neoplastic tissues samples compared to normal tissues, a result which is compatible with tumor-associated replication and transgene expression, again in line with animal data.<sup>18,44</sup>

While viral DNA was detected in many samples, none of these were found to express adenoviral hexon antigen, when the tissues were examined by immunohistology (IH). These results may reflect the short time window between hexon expression and cell destruction, mediated by either oncolysis or killing by immune cells, or both. However, IH has a relatively low sensitivity compared to PCR, and the amount of hexon antigen within the human tissue samples may be below detection level. When comparing the IH results from the infected cell pellet (analyzed at 48 hours) and an injected tumor in a mouse (analyzed at 72 hours), a major quantitative difference is already obvious, within the number of positive cells and the staining intensity, indicating a substantially lower amount of virus in the tumor (**Supplementary Figure S1**). In contrast to high-dose “synchronized” infection necessarily utilized in the hexon staining optimization assay (when samples were analyzed 24 hours after infection), the natural serendipitous and opportunistic progression of infection in human tumors may result in much lower amounts of hexon at any given time point. As autopsy was not performed immediately after virus injection, the highest synchronized hexon expression peak may have been at an earlier time point. The time window between hexon expression and cell death might also be small, especially in the scenario of pre-existing or induced antiadenoviral immunity, which would be expected to enhance clearance of infected cells. These aspects may indicate limited utility of hexon detection in the context of oncolytic adenoviruses in humans, and indeed we are unaware of examples where the assay would have been positive. Alternatively,

immunohistochemical staining for adenoviral E1A protein could be a potentially valuable tool in future work if staining protocols for adenoviruses with the 24bp deletion in the E1A region in human tissues are successfully established.

In summary, analysis of autopsy specimens from patients who had been treated with oncolytic adenoviruses, and later died due to tumor progression, indicated systemic spread of the injected virus to many organs and noninjected tumors. Our data suggest that oncolytic adenoviruses are able to disseminate through the vascular route from injected tumors following replication and lysis and/or following intravenous injection. Importantly, this sets the stage for intravenous delivery of oncolytic adenovirus in human trials. Also, it provides mechanism of action data, supporting preclinical hamster data, which has shown vascular dissemination to distant tumors following injection into local tumors.<sup>45</sup> A particularly interesting finding was the transduction of brain tissue through the vascular route. As patients with brain metastases represent the most difficult clinical situation in oncology, it could be of clinical relevance that oncolytic adenoviruses are able to transduce the central nervous system through the vascular route, without major neurological symptoms.

## MATERIALS AND METHODS

**Oncolytic virus treatments.** The viruses used for treatment were produced on a nontranscomplementing cell line (human lung adenocarcinoma epithelial cell line A549), to avoid risk of recombination back to a wild-type E1A. Virus production was done by Oncos Therapeutics (Helsinki, Finland), and regulated by the Finnish medical authority FIMEA according to the guidelines detailed below. Viral preps were screened for purity and quality including absence of wild-type contamination.

Oncolytic adenovirus treatments were given in the context of an ATAP.<sup>46,47</sup> Patients had signed a written informed consent form and treatments were administered according to the Declaration of Helsinki and Good Clinical Practice. ATAP is in compliance with EU and Finnish regulations and the program is under regulation of the Finnish Medical Agency as determined by EU/1394/2007. Data for this study were collected and analyzed retrospectively, with positive statements from the Helsinki University Central Hospital ethics committee (Dnro 313/13/03/02/2012) and the Finnish National Supervisory Authority for Welfare and Health (Dnro 2797/06.01.03.01/2013). While ATAP was an individualized approach, generally only patients with solid tumors refractory to conventional therapies and progressive thereafter, WHO performance score  $\leq 3$ , no other severe disease and no major organ function deficiencies or organ transplants, HIV or other major immunosuppression, were eligible to receive treatments.<sup>16–18,20,25,31–33,37,45</sup> Patient characteristics are provided in [Table 1](#).

Patients ( $n = 11$ ) received their virus treatments by ultrasound-guided intratumoral injection, usually combined with an intravenous bolus. Patients with peritoneal and pleural involvement received part of the virus dose as an injection into the respective body cavity. Treatments were given at Docrates Hospital as single treatments or in a series of three treatment cycles approximately 3 weeks apart. The virus vectors, Ad5/3-Cox2L-D24, Ad5-D24-RGD, Ad5-D24-RGD-GMCSF, ICOVIR-7, Ad5-D24-GMCSF, Ad5/3-D24-GMCSF, Ad5/3-E2F-D24-GMCSF and Ad5/3-hTERT-CD40L, and the treatment protocols have been published previously.<sup>17,18,20,25,31–34</sup> Treatments are detailed in [Table 1](#). Adverse reactions were monitored for 28 days and recorded according to Common Terminology Criteria for Adverse Events v3.0, and are listed in [Supplementary Table S1](#). Patient monitoring included routine laboratory tests. Blood cell counts at baseline and during adenoviral treatments are shown in [Supplementary Table S2](#).

Neutralizing antibody titering against the used adenoviral vectors was done as described earlier.<sup>34</sup> Depending on the virus that the patients had received in treatment, different replication-deficient adenoviruses were used for titration to ensure identical match of virus capsid: Ad5luc1 for Ad5-D24-GM-CSF, Ad5/3luc1 for Ad5/3-D24-GM-CSF and Ad5lucRGD for Ad5-RGD-D24 and Ad5-RGD-D24-GM-CSF. The neutralizing antibody titer was determined as reciprocal of the lowest degree of dilution that blocked gene transfer  $>80\%$ . For patients with available samples, neutralizing antibody titers at baseline and during adenoviral treatments are shown in [Supplementary Table S3](#).

**Autopsies.** According to Finnish law and medical custom, the need for autopsy was determined by the patient's attending physician at the place of death, and this decision was independent from decisions relating to cancer treatment. Autopsies were performed at University Hospital pathology departments or at The Department of Forensic Medicine at the Hjelt Institute (University of Helsinki and National Institute for Health and Welfare). Formalin-fixed tissue samples were prepared by the pathologists as part of the autopsy. In some cases, tissue samples from different organs had been placed together in one paraffin block, as part of their routine protocols. Also, while for most cases, one sample per tissue was collected, in others, several samples ( $n = 2–15$ ) from the same organ, especially tumors, were sampled from multiple sites. In three cases, cryo-preserved tissue samples snap frozen immediately after collection and stored in  $-80^{\circ}\text{C}$ , were available as well.

**DNA analysis.** DNA was extracted from formalin-fixed and paraffin wax embedded tissue samples, archived as a routine part of the autopsy, using the QIAamp DNA FFPE Tissue kit (Qiagen, Helsinki, Finland). Quantitative real-time PCR (qPCR) was performed with primers and probe for the adenoviral E1A gene with the 24bp deletion that renders the virus tumor-selective (forward primer: 5'-TCCGGTTTCTATGCCAACCT-3'; reverse primer: 5'-TCCTCCGGTGATAATGACAAGA-3' and probe: 5'-FAM-TGATCGATCCACCCAGTGA-3'-MGBNFQ). In addition, a probe complementary to a sequence included in the wild-type E1A region was used to test the samples for the presence of wild-type adenovirus (probe: 5'-VIC-TACCTGCCACGAGGCT-3'-MGBNFQ). For one patient, who had been treated with Ad5/3-hTERT-CD40L, an oncolytic virus without a 24bp deletion in E1A, PCR amplification was based on primers and probe targeting the E3 region flanking the CD40L transgene (forward primer 5'-CCGAGCTCAGTACTCCATC-3', reverse primer 5'-GCAAAAAGTGCTGACCCAAT-3' and probe 5'-FAM-CCTGCCGGG AACGTACGATG-3'-MGBNFQ). Human  $\beta$ -actin primers and probe (forward primer 5'- TCACCCACACTGTGCCCATCT -3', reverse primer 5'- GTGAGGATCTTCATGAGGTAGTCTCAGTC -3' and probe 5'-FAM-ATGCCCTCCCCATGCCATCCTGCGT-3') served as internal control and to normalize viral DNA copies per amount of genomic DNA.

The quantitative real-time PCR conditions for each 25  $\mu\text{l}$  reaction were: 2 $\times$  LightCycler480 Probes Master Mix (Roche, Mannheim, Germany), 800 nmol/l of each forward and reverse primer, 200 nmol/l of each probe, and 250 ng of extracted DNA. PCR reactions were carried out in a LightCycler (Roche) with the following cycling conditions: 10 minutes at  $95^{\circ}\text{C}$ , 50 cycles of 10 seconds at  $95^{\circ}\text{C}$ , 30 seconds at  $62^{\circ}\text{C}$ , 20 seconds at  $72^{\circ}\text{C}$ , and 10 minutes at  $40^{\circ}\text{C}$ . TaqMan exogenous internal positive control reagents (Applied Biosystems, Espoo, Finland) were used in the same PCR runs in order to test each sample for the presence of PCR inhibitors. Samples were run in duplicates ( $\beta$ -actin) or triplicates (adenoviral genes).

Regression standard curves for E1A copies were generated using adenoviral plasmid DNA serially diluted from  $1 \times 10^9$  copies to 1 copy. The standard curve for wild-type E1A was generated similarly, from E1A-wild-type plasmid (GeneArt Life Technologies, Germany). The standard curve for human  $\beta$ -actin was established with known amounts (800–0.08 ng) of DNA extracted from cultured cells. Cycle threshold values were plotted on the standard curves to determine the actual DNA copy number and the number of adenoviral copies per ng genomic DNA was subsequently calculated.



For serum samples, DNA extraction and real-time PCR were performed as previously described.<sup>34</sup> The viral loads in fluids were calculated using a regression standard curve based on serial dilutions of pAd5-D24-GM-CSF plasmid DNA ( $1 \times 10^9$  to  $1 \times 10$ ) in normal human serum from healthy donors. Positive samples were confirmed by real-time PCR using LightCycler480 SYBR Green I Master mix (Roche) and primers specific for sequences for adenovirus, GM-CSF, Cox2L, RGD, and adenovirus fiber knob 3.<sup>18,20,33,34</sup>

Each reaction was assessed for the lower limit of reliable detection and only results above that threshold were considered positive. The limit of quantification for the E1A reaction was at CP 38.495, as assessed by spiking serum samples with increasing adenovirus titers, corresponding to 29.85 viral DNA copies per 25  $\mu$ l reaction volume and 500 viral particles per ml of serum. For CP values higher than this, titers were extrapolated using a standard curve. The limit of detection for the 24bp deleted E1A was at CP 42.11, corresponding to 2.89 viral DNA copies per reaction volume. The limit of detection for the wild-type E1A reaction was at CP 40.00 corresponding to 13.2 adenoviral DNA copies per 25  $\mu$ l reaction volume.

**Adenoviral hexon staining.** Immunohistology was performed using a monoclonal mouse anti-human adenovirus hexon (ATCC strain VR847 clone BO 25, Acris Antibodies GmbH, Herford, Germany) and the peroxidase anti-peroxidase method as previously described.<sup>48</sup> Briefly, formalin-fixed, paraffin-embedded tissue sections (3–5  $\mu$ m) were pre-treated with bacterial protease for antigen retrieval and then incubated with the primary antibody (1:100 in Tris-buffered saline Tween) at 4 °C overnight, followed by rat anti-mouse IgG and mouse peroxidase anti-peroxidase (Jackson Immuno Research, Suffolk, UK), and 3,3'-diaminobenzidine to visualize the reaction.

Formalin-fixed and routinely paraffin wax embedded A549 human lung adenocarcinoma cell pellets, collected 48 hours after they had been infected with Ad5-D24E3<sup>49</sup> at 100 and 1,000 VP per cell, were used as positive controls and an uninfected cell pellet as a negative control. Furthermore, one young male nude/NMRI mice was obtained from Harlan (Indianapolis, IN) and human fibrosarcoma HT-1080 cells ( $5 \times 10^6$  cells/tumor) were injected subcutaneously at four different sites on the back of the mouse. When tumors reached the size of approximately 5 mm diameter, Ad5/3-D24-GM-CSF virus diluted in saline was injected intratumorally ( $1 \times 10^9$  VP/tumor). Three days postinfection tumors were collected and routinely formalin-fixed and paraffin wax embedded. This tumor served as an *in situ* positive control, with sections incubated with Tris-buffered saline Tween without the primary antibody serving as additional negative controls. The animal protocol was reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

**Virus culture and GM-CSF measurements.** Routine diagnostic virus culture was performed on the cryo-preserved tissue samples at an independent commercial laboratory (HUSLAB, Helsinki, Finland). Positive samples were confirmed by real-time PCR for the E1A gene as described above and by using LightCycler480 SYBR Green I Master mix (Roche) and primers specific for adenovirus 3 fiber (forward primer: 5'-AGCGTATCCATTTGTCCTTCC-3', reverse primer: 5'-GGTTATGAGGGTGCCTGAGT-3') and COX2L-promoter sequences (forward primer, 5'-CACGTCCAGGAACCTCCTCAG-3' and reverse primer 5'-CGGCCATTCTTCGGTAATA-3').

For measuring the level of human GM-CSF, cryo-preserved tissue samples were minced with a scalpel and 50mg incubated with 5  $\mu$ l of protease inhibitor (P8340, Sigma-Aldrich, Helsinki, Finland) and 500  $\mu$ l of digestion mixture consisting of RPMI 1640 medium with 10 mmol/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and 1.6 mmol/l phenylmethylsulfonyl fluoride (Sigma-Aldrich), 40  $\mu$ g/ml gentamycin (Amresco, Solon, OH), 100  $\mu$ g/ml bovine serum albumin (Sigma-Aldrich)

and 100  $\mu$ g/ml Zwittergent 3–12 (Merck4Biosciences, Darmstadt, Germany). After incubation for 90 minutes at 37 °C under continuous agitation the digestates were subjected to 30 seconds of sonication and centrifuged at 2,000g for 10 minutes at 4 °C. Supernatants were collected and stored at –80 °C until used in the FACSArray. GM-CSF concentrations in tissue digestion supernatants and serum samples were measured using the Cytometrin Bead Array Soluble Protein Master Buffer Kit and Cytometrin Bead Array Human GM-CSF Flex set (Becton Dickinson) according to the manufacturer's instructions and the LSRFortessa cell analyzer (Becton Dickinson).

**Statistics.** Statistical correlations were analyzed with PASW Statistics 18.0 Software, using the Fisher's exact test and two-tailed test for Pearson's correlation coefficient.

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## SUPPLEMENTARY MATERIAL

**Figure S1.** Immunohistochemical staining for adenovirus hexon with a monoclonal antibody.

**Table S1.** Adverse reactions recorded within 28 days of each treatment.

**Table S2.** Blood cell counts at baseline and after virus treatments.

**Table S3.** Neutralizing antibody titers after virus treatments.

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